Article[+] Supplement

A gene regulatory network controls the binary fate decision of rod and bipolar cells in the vertebrate retina

Sui Wang, Cem Sengel, Mark M. Emerson, and Constance L. Cepko

Introduction

With the recent advance of the CRISPR/Cas9 method for RNA-guided genome engineering, targeted alterations of genomic DNA have become more rapid and accessible, even for non-model organisms (Cong et al., 2013; Mali et al., 2013; Sternberg et al., 2014). As the removal of a DNA element from the genome provides a strong test of its function, we wished to use CRISPR/Cas9 to delete the B108 enhancer from the genome, to determine if it is required for *Blimp1* regulation. In the past, for experiments in mice, genomic deletions could be accomplished using classical mouse genome engineering methods. Classical methods require the manipulation and screening of embryonic stem (ES) cells, injection of successfully engineered cells to blastocysts, implantation into a pseudopregnant female, a 3-week gestation, and screening of the progeny for successful contribution of the ES cells to the tissue of interest, or for germ line transmission. Even though CRISPR/Cas9 reagents can be injected directly into fertilized eggs (Wang et al., 2013; Yang et al., 2013), obviating the need for the ES stage of the work, the cost, infrastructure, and time required for the

generation, screening and breeding of engineered mice still makes this a low throughput method. In contrast, acute delivery methods, such as electroporation or viral transduction, can deliver CRISPR/Cas9 constructs directly to tissues, rapidly and without the need for the infrastructure and skill required for the classical methods. We thus explored the efficiency and accuracy of CRISPR/Cas9 using electroporation *in vivo* in the mouse retina.

Design and construction of CRISPR/Cas9 plasmids

To obtain CRISPR/Cas9 constructs that target genomic sequences, the Px330 vector, created by the laboratory of Dr. Feng Zhang, and obtained from Addgene (Plasmid 42230), was used. Detailed information about this vector can be found at: http://www.genome-engineering.org/crispr/?page_id=23. Px330 encodes the Cas9 nuclease, driven by a broadly active promoter, CBh, as well as harbors a cloning site for the insertion of an oligonucleotide for targeting the genomic site of interest. When the genome targeting sequence (20bp) is cloned into Px330, a guide RNA (gRNA) is transcribed from the human U6 PolIII promoter. The gRNA is a single RNA comprising the genome targeting sequence and the Cas9 tracrRNA sequence. This gRNA brings Cas9 to the genomic DNA target, where it makes a double-strand break at the targeted site. To generate targeted deletions in the genome, two gRNAs, and thus 2 CRISPR/Cas9 constructs, are designed to make two double-strand DNA breaks flanking the sequence to be deleted, with some constraints regarding the exact sequence to be targeted, as discussed below. The DNA that is cleaved by Cas9 is typically ligated by the endogenous

non-homologous end joining (NHEJ) repair pathway, which results in a targeted deletion or disruption in the genome.

The following steps were taken to create the CRISPR/Cas9 plasmids used in this study (Figure P1).

1. Search for genome target sequences. As Cas9 has a preference for a particular sequence, the protospacer adjacent motif, or PAM, one must search for this sequence near the desired cleavage sites. For S. pyogenes Cas9 nuclease, which is the one encoded in Px330, the PAM is “NGG”.

2. Design two complementary oligonucleotides of 20bp 3’ to the “NGG” (indicated by “n” below) with overhangs (“uuu…” is the reverse complement sequence of “nnn…”).

For example: Oligo1: 5’- *CACCGnnnnnnnnnnnnnnnnnnn*;

Oligo2: 5’- *AAACuuuuuuuuuuuuuuuuuuuC.*

3. Anneal Oligo1 and Oligo2 (100μM) by adding 1-2μl of each oligonucleotide into 50ul annealing buffer (50mM TrisHCl, 100mM NaCl, pH 7.4), and leaving the mix at 95°C for 5min, 60°C for 10min.

4. Treat annealed oligonucleotides with T4 Polynucleotide Kinase (PNK) (NEB, catalog number: M0201S). Specifically, mix 5μl annealed products, 2μl T4 DNA ligase buffer, 1μl PNK and 12μl H_2O, and then leave this at 37°C for 30min, 70°C for 10min. Purification is not necessary after PNK treatment.

5. Ligate PNK-treated annealed products with BbsI digested Px330 plasmid.

For additional and alternative protocols, please refer to (Ran et al., 2013).

Electroporation of plasmids into the mouse retina *in vivo*

We have developed a protocol to efficiently deliver plasmids into the mouse retina *in vivo*, *via* injection of plasmid solutions into the subretinal space and the application of short pulses of voltage from an electroporator (Matsuda and Cepko, 2004). This method primarily delivers the plasmids to the mitotic RPCs, which line the subretinal surface of the retina, adjacent to the site of plasmid injection. The injected plasmids likely do not integrate, but are passed on to daughter cells of each cell division, eventually diluting out if there are many cell divisions. In the case under study here, most daughter cells are generated within 1-3 cell cycles, and they show expression of injected plasmids for at least 70 days post-electroporation (Chen and Cepko, 2009). The number of plasmids taken up by each electroporated cell is not known, but must be a relatively large number, as 4 plasmids co-electroporated show evidence of expression in the majority of electroporated cells (Tang et al. 2013).

Claudio Punzo (now at University of Massachusetts Medical School) and Rahul Kanadia (now at University of Connecticut), while in the Cepko Lab, optimized this protocol by utilizing a Femtojet Express pressure injector (Eppendorf; 920010521) and pulled glass micropipets (Humagen, catalog number: C060609) to deliver the DNA. In the original protocol, small incisions needed to be made in the eye to enable a Hamilton syringe with a blunt-ended 32 or 33-gauge needle to reach the subretinal space. This often damaged the retina. In the current

protocol, sharp-ended glass micropipets are used, in place of a Hamilton syringe and needle. These sharp micropipets pass through the sclera and retinal pigment epithelium (RPE) layers to reach the subretinal space, without the need for an incision. Detailed information about this protocol is shown in Figure P2.

Test of the CRISPR/Cas9 efficiency in tissues *in vivo*

As we wished to create a targeted deletion of B108 in the genome, two gRNAs, and thus 2 CRISPR/Cas9 constructs, were designed to direct the Cas9 nuclease to two cleavage sites that flank B108. In order for this to be an effective strategy, the efficiency and specificity of CRISPR/Cas9-medicated cleavage must be sufficient for the assay that is used. We thus first investigated the efficiency of CRISPR/Cas9 to achieve targeted deletions in retinal tissues *in vivo* using a reporter mouse strain.

The mouse strain, *Rosa*$^{mTmG/mTmG}$ (The Jackson Laboratory, Stock number: 007576) (Muzumdar et al., 2007) (Supplemental Figure 3), affords a straightforward assessment of the efficiency of deletion of a genomic element. This strain expresses membrane Tdtomato (mTdtomato), which when deleted, leads to expression of a membrane GFP (mGFP). The strain was designed as a Cre-reporter, as mTdtomato is flanked by LoxP sites. We designed CRISPR/Cas9 plasmids that target sites near both LoxP sites in *Rosa*$^{mTmG/mTmG}$ mice. By quantifying the number of cells that express mGFP, we could measure the efficiency of CRISPR/Cas9 in achieving targeted deletions in retinal tissues *in*

vivo. Specifically, the following steps were taken (see also Supplemental Figure 3).

1. CRISPR/Cas9 plasmids targeting the *Rosa*$^{mTmG/mTmG}$ allele were generated as described in the "Design and construction of CRISPR/Cas9 plasmids" section. In the *Rosa*$^{mTmG/mTmG}$ allele, the sequences (17bp) 3' to the two LoxP sites are identical. Thus, we designed CRISPR/Cas9 constructs targeting this 17bp region and the LoxP sequence, so that one CRISPR/Cas9 construct could cleave near both LoxP sites and result in the deletion of the mTdtomoto cassette. Two different oligonucleotides were designed to target genomic sites near the two LoxP sites in *Rosa*$^{mTmG/mTmG}$ mice. Only one, namely CRISPRmTmG, worked, and thus one should design multiple CRISPR/Cas9 plasmids targeting the same genomic regions, and then test their efficiency to find the construct that is adequate for one's assays.

2. CRISPR/Cas9 plasmids (CRISPRmTmG) were injected and electroporated into P0 retinas *in vivo* as described in the "Electroporation of plasmids in the mouse retina *in vivo*" section, together with a CAG-LacZ plasmid. The CAG-LacZ plasmid served as an electroporation efficiency control. Due to the high co-delivery efficiency of multiple plasmids *via* electroporation into the retina (Matsuda and Cepko, 2004), most retinal cells which received the CAG-lacZ plasmid should have received the CRISPRmTmG plasmid as well.

3. Twenty-one days after electroporation, we sacrificed animals and harvested their retinas. By analyzing the percentage of $LacZ^+$ retinal cells that turned on mGFP expression, we obtained a rough idea of the general efficiency of CRISPR/Cas9 in retinal tissues *in vivo*. Another advantage of the dual reporters in this mouse strain is the ability to assess homozygotes for deletion of Tdtomato from both alleles, as cells that only deleted one allele would be both $Tdtomato^+$ and $mGFP^+$. We found that a significant number of retinal cells that expressed mGFP did not express mTdtomato (Supplemental Figure 3B), suggesting CRISPR/Cas9 plasmids can achieve homozygous targeted deletions.

The CRISPR/Cas9 efficiency may vary significantly at different loci due to differences in chromatin structure. The above experiment showed the CRISPR/Cas9 efficiency at the *Rosa* locus in retinal cells *in vivo*. However, as this is a new method and relatively few genomic loci have been targeted, it is not known how variable the efficiency is. Similarly, different cell types may have different efficiencies, e.g. postmitotic cells may have a lower efficiency than cells that are replicating their genomic DNA (Hsu et al., 2014).

Deletion of the B108 enhancer from the mouse genome

We investigated whether CRISPR/Cas9 could be utilized to delete the B108 enhancer from the mouse genome in the retina *in vivo* using electroporation. The following steps were taken to address this question (see also Figure 3).

1. Design and construct CRISPR/Cas9 plasmids.

As indicated in Figure 3A, two oligonucleotides targeting the genomic region 5' to B108 and two oligonucleotides targeting the genomic region 3' of B108 were designed and constructed.

2. Test the deletion efficiency of different combinations of CRISPR/Cas9 plasmids (see also Figure 3B).

To test which combinations of the 5' and 3' CRISPR/Cas9 plasmids could achieve the best deletion efficiency, we electroporated different combinations of CRISPR/Cas9 plasmids into P0 mouse retinas, together with the CAG-EGFP plasmid, which served as the electroporation efficiency control. Five days later, retina tissues were dissociated as previously described (Trimarchi et al., 2008) and the EGFP+ cells were collected by FACS sorting. Genomic DNA was prepared from these cells by the QuickExtract DNA Extraction Solution (Epicentre, Catalog number: QE0905T), and was subjected to PCR using oligonucleotides flanking the B108 sequence. A smaller PCR fragment, the size predicted for the B108 deletion, should be observed only when this enhancer was deleted (The L band in Figure 3B). The 5' and 3' CRISPR/Cas9 plasmid pair which gave rise to a strong L band was selected for future experiments.

3. Tracking the fates of cells following CRISPR/Cas9 deletion.

In order to assess whether a cell suffered the loss of Blimp1 function due to the deletion of B108, we designed an experiment that utilized Cre and reporter

plasmids. The *Blimp1* conditional knockout (CKO) mice have been shown to have an increase in bipolar cells and a decrease in rod photoreceptors (Brzezinski et al., 2010; Katoh et al., 2010). Thus, we designed an experiment to assay whether more bipolar cells were generated when cells were transduced with the CRISPR/Cas9 B108 targeting plasmids, which would indicate that B108 led to a loss of expression of the endogenous Blimp1 gene. This experiment was based upon the B108-Cre fate mapping plasmid and the Chx10 dual color reporter plasmid that is only expressed in bipolar cells.

i). To report bipolar identity, we constructed the Cre-sensitive reporter plasmid, Chx10BP-LCL-EGFP. In the absence of Cre, electroporated bipolar cells would be labeled by mCherry. Following Cre activity, electroporated cells would lose mCherry and would instead be labeled by EGFP (see also Supplemental Experimental Procedures for more details).

ii) To trace cells with B108 activity, we constructed a B108-Cre construct (see also Supplemental Experimental Procedures for more details). We electroporated the Chx10BP-LCL-EGFP and B108-Cre plasmids into P0 retinas *in vivo* and harvested them at P14. As shown in Figure 3F, almost every electroporated bipolar cell was labeled by mCherry, as they did not have a history of activating B108-Cre. In contrast, only electroporated rods showed that they had a history of B108-Cre expression (Figure 2G and 2H).

iii) We co-electroporated CRISPR plasmids that target B108, along with Chx10BP-LCL-EGFP and B108-Cre plasmids, into P0 retinas *in vivo*. The B108-Cre plasmid did not encode the CRISPR/Cas9 target sites, and thus would be expected to retain the activity shown in the control experiment, i.e. label only rods, unless loss of the B108 enhancer from the mouse genome led to a loss of Blimp1 expression. If deletion of the B108 sequence from the mouse genome created a loss of Blimp1, then a portion of the CRISPR/Cas9 electroporated cells fated to be rods, and thus marked by B108-Cre, should behave as they did in the Blimp1 CKO retinas, i.e. become bipolar cells. This is exactly what was observed, as there was a significant increase in the number of $EGFP^+$ bipolar cells (Figure 3F-G). To provide an indirect measure of the efficiency of the B108 deletion, the number of mCherry+ and EGFP+ bipolar cells were counted. In the Blimp1 CKO retinas, many rods remain, and the additional bipolar cells, generated by the fate change from rods, die over time. This makes it difficult to determine the expected frequency of cells undergoing the rod to bipolar fate change for B108-deleted cells. Nonetheless, a 5-6 fold increase was seen in the ratio of $EGFP^+$ cells over all electroporated bipolar cells following the CRISPR/Cas9 electroporation, indicating that B108 is required for normal Blimp1 expression. The level of increase in $EGFP^+$ bipolar cells suggested that the CRISPR/Cas9 mediated deletion efficiency was substantial, likely approaching the frequency seen in the experiment described above for the *Rosa*$^{mTmG/mTmG}$ mouse, in which about 50% of the electroporated cells showed deletion of the targeted site.

References

Brzezinski, J., Lamba, D., and Reh, T. (2010). Blimp1 controls photoreceptor versus bipolar cell fate choice during retinal development. Development (Cambridge, England) *137*, 619-629.

Chen, B., and Cepko, C.L. (2009). HDAC4 regulates neuronal survival in normal and diseased retinas. Science (New York, N.Y.) *323*, 256-259.

Cong, L., Ran, F., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P., Wu, X., Jiang, W., Marraffini, L.*, et al.* (2013). Multiplex genome engineering using CRISPR/Cas systems. Science (New York, N.Y.) *339*, 819-823.

Hsu, P.D., Lander, E.S., and Zhang, F. (2014). Development and Applications of CRISPR-Cas9 for Genome Engineering. Cell *157*, 1262-1278.

Katoh, K., Omori, Y., Onishi, A., Sato, S., Kondo, M., and Furukawa, T. (2010). Blimp1 suppresses Chx10 expression in differentiating retinal photoreceptor precursors to ensure proper photoreceptor development. The Journal of neuroscience : the official journal of the Society for Neuroscience *30*, 6515-6526.

Mali, P., Yang, L., Esvelt, K., Aach, J., Guell, M., DiCarlo, J., Norville, J., and Church, G. (2013). RNA-guided human genome engineering via Cas9. Science (New York, N.Y.) *339*, 823-826.

Matsuda, T., and Cepko, C. (2004). Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proceedings of the National Academy of Sciences of the United States of America *101*, 16-22.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis (New York, N.Y. : 2000) *45*, 593-605.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nature protocols *8*, 2281-2308.

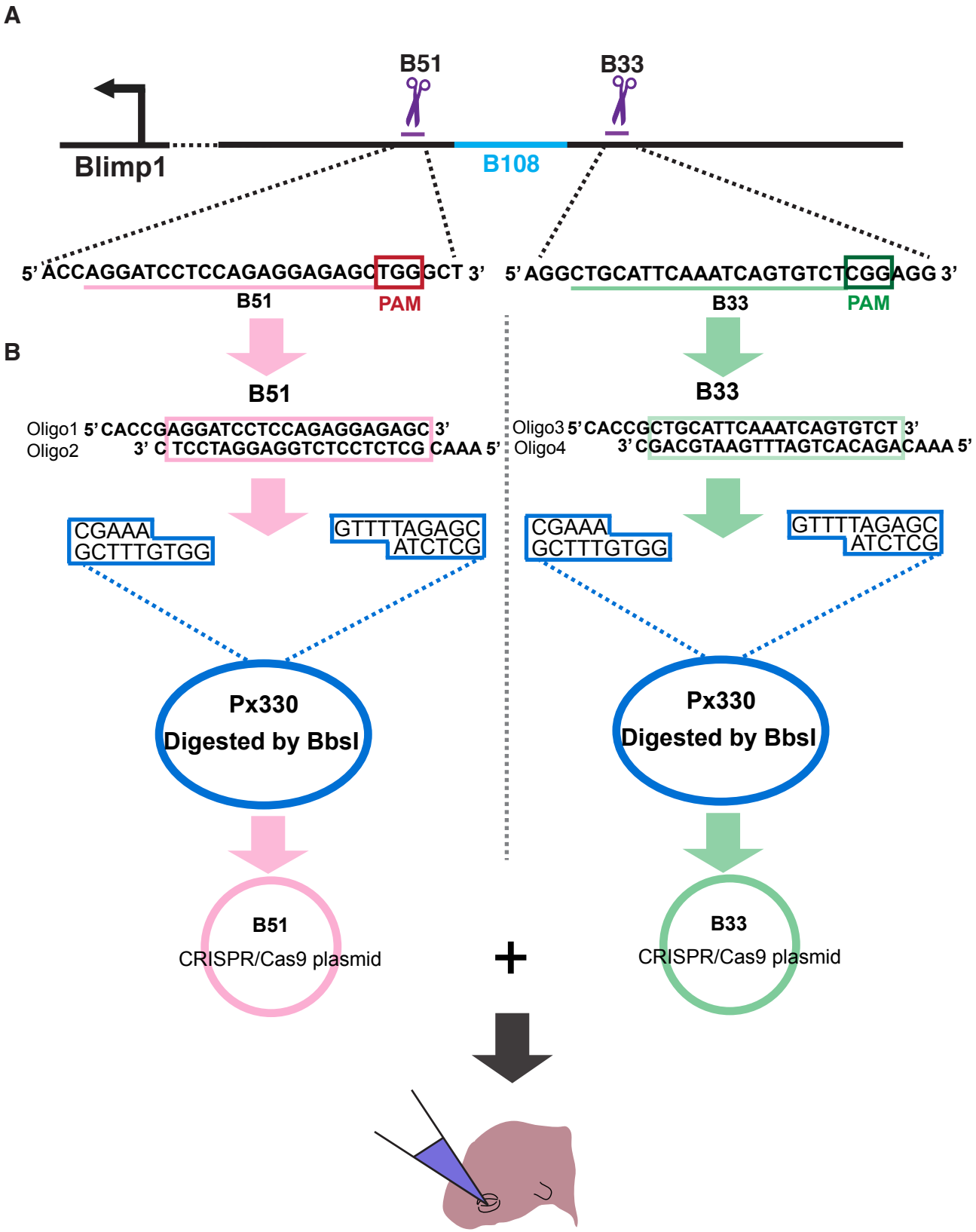
Sternberg, S.H., Redding, S., Jinek, M., Greene, E.C., and Doudna, J.A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature *507*, 62-67.

Trimarchi, J., Stadler, M., and Cepko, C. (2008). Individual retinal progenitor cells display extensive heterogeneity of gene expression. PloS one *3*.

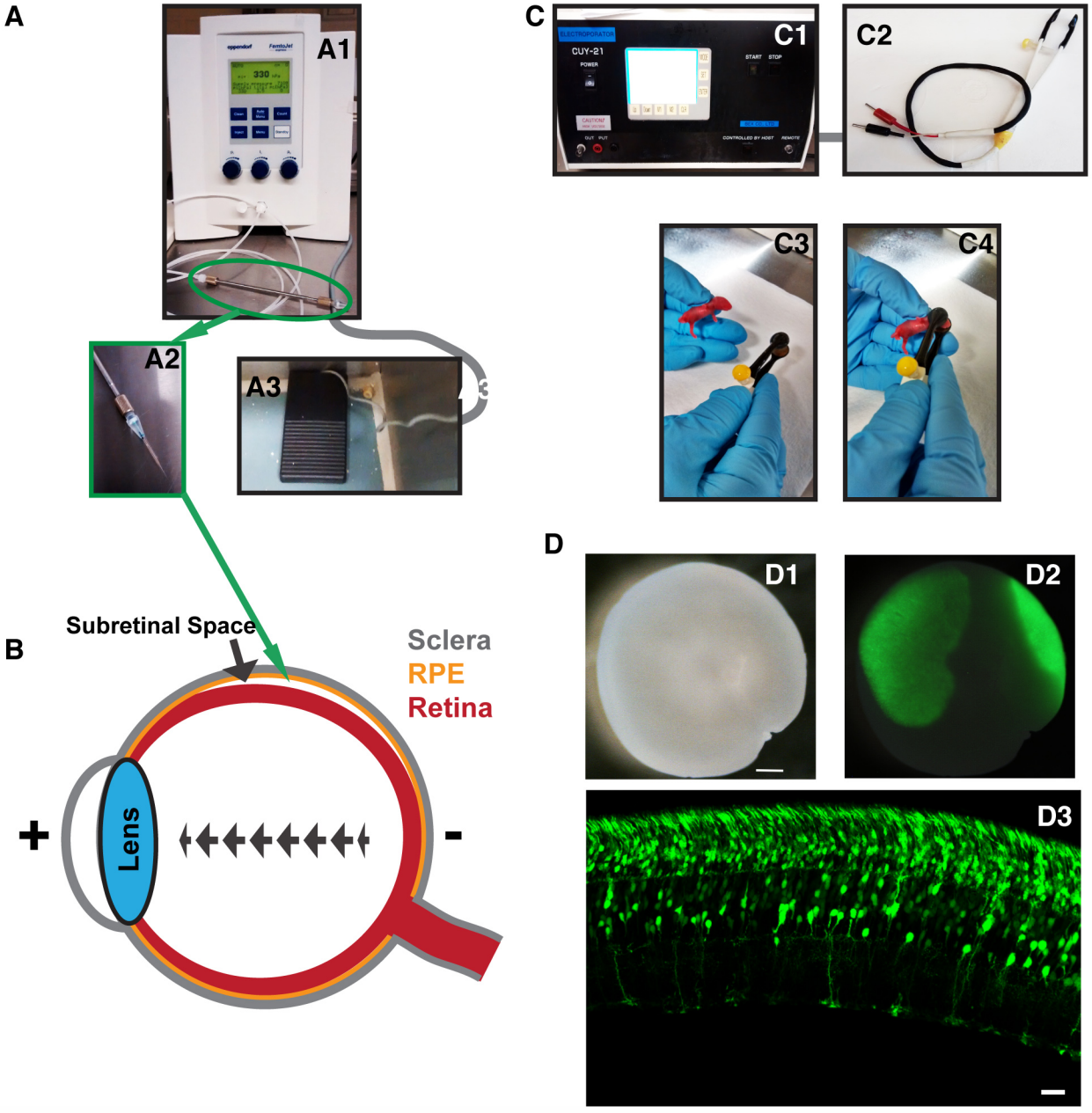
Wang, H., Yang, H., Shivalila, C.S., Dawlaty, M.M., Cheng, A.W., Zhang, F., and Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell *153*, 910-918.

Yang, H., Wang, H., Shivalila, C.S., Cheng, A.W., Shi, L., and Jaenisch, R. (2013). One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell *154*, 1370-1379.

Article+ Supplement Figure P1



Article+Supplement Figure P2



Article[+] Supplement Figure Legend

Figure P1. Design and construction of CRISPR/Cas9 plasmids.

(A) The *Blimp1* locus. The B108 enhancer is highlighted in light blue. The target sites, targeted by the B51 and B33 oligonucleotides, along with the associated PAM sequences, are shown. **(B)** Construction of the B51 and B33 targeting plasmids. Oligo1-2: complementary oligonucleotides of the 20bp B51 target sequence. Oligo3-4: complementary oligonucleotides of the 20bp B33 target sequence. The Px330 plasmid digested with BbsI is shown in blue. The CRISPR/Cas9 B51 or B33 plasmids were generated by ligating annealed Oligo1 and Oligo2 or Oligo3 and Oligo4 into BbsI digested Px330 vector. B51 and B33 plasmids were mixed in a 1:1 ratio and electroporated into P0 retinas *in vivo*, to delete B108 from the genome.

Figure P2. Delivery of plasmids into the mouse retina *in vivo* by electroporation.

(A) The apparati used for injection of plasmid DNA solutions into the subretinal space. **A1**. Eppendorf Femtojet Express (Cat No: 920010521); Capillary holder (Eppendorf, Cat No: 920007392); Positioning aids (the metal part, Eppendorf, Cat No: 920005829). The working parameters for Femtojet are: Pressure: 330 hPa & Time: 3.5 seconds. At this setting, about 0.3-0.5µl of solution is delivered into the subretinal space each time the foot pedal is pressed. **A2**: glass micropipet needles (Humagen, catalog number: C060609). The micropipet is connected with the Femtojet express capillary holder. Plasmid DNA solutions are

prepared as described previously (Matsuda and Cepko, 2004). To load plasmid DNA solutions into the micropipet, microloaders (Fisher Scientific, Cat No: E5242956003) are used. Eight to ten μl of DNA solution are loaded into a micropipet for each plasmid mixture. **A3**. Foot control pedal (Eppendorf, Cat No: 920005098). **(B)** Schematic of the mouse eyeball. The subretinal space (black arrow) is indicated, with the green arrow indicating the target location for the injection. "+": the position of the positive electrode. "-" : the position of the negative electrode. Arrows inside the eyeball indicate the direction of DNA flow during electroporation. **(C)** The apparati used for electroporation. **C1**: Electroporator: CUY21EDIT Electroporator, (available from BEX Co., LTD. or Nepagene, both located in Japan, Cat No: CUY21EDIT). ECM830 (BTX) (Harvard Apparatus, Cat No: 450052) works as well. The working parameters are: Volts: 80V; Pulse-on: 50ms; Pluse-Off: 950ms; Number of Pluses: 5. **C2**: Tweezer-type electrodes (Harvard Apparatus, BTX, model 520, 7mm diameter, Cat No: 450165). **C3-4**: electroporation of newborn mouse pups with the tweezer-type electrodes. **(D)** Representative retinal images after electroporation of plasmids in the retina *in vivo.* The CAG-EGFP plasmid was injected and electroporated into P0 retinas *in vivo* as described in panel A-C. Fourteen days later, retinas were harvested. **D1-D2**: whole mount retinas without any signal amplification. Scale bar: 150μm. **D3**: section view of the $EGFP^+$ electroporated retinal cells. Scale bar: 10μm.